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13. ABSTRACT ***(Maximum 200 Words)***

Citrus limonoids are a class of chemically related compounds present in lemon, lime, orange and grapefruit. We have shown that nomilin and a limonoid glucoside mixture are potent inhibitors of prolifertation of estrogen receptor-negative (ER-) and –positive (ER+) human breast cancer cells in culture. In the present experiments, we tested deacetylnomilin, deoxylimonin, ichangin, isoobacunoic acid, limonol, limonin carboxymethoxime, methyl deoxylimonate, methylnomilinate,methyl deacetylnomilinate, methyl isolimonate, nomilin glucoside, nomilinic acid glucoside, 7a-obacunol, obacunone and obacunone glucoside for their ability to inhibit the proliferation of MDA-MB-435 ER- and MCF-7 ER+ human breast cancer cells. In ER- cells, limonin methoxime and deacetylnomilin were the most effective inhibitors having IC50s of 0.02 and 0.07 ug/mL respectively. In ER+ cells, deacetylnomilin, obacunone and methyl nomilinate were the most effective inhibitors of proliferation having IC50s of 0.005, 0.009 and 0.01 ug/mL respectively. Maximum tolerated dose studies were conducted in nude mice for limonin, nomilin and the glucoside mixture. We found that this dose was 1% of the diet for nomilin, 2% of the diet for limonin and 4% of the diet for the glucoside mixture. These results suggest that citrus limonoids have important anti-cancer activity.

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✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

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Introduction

Breast cancer is the second most common cause of cancer related deaths in North American women (1). Modern preventative and treatment methods are limited and the investigation of natural, low toxicity dietary components for their anti-cancer properties is of great interest . There is general agreement that plant-based diets rich in whole grains legumes , fruits and vegetables, reduce the risk of various types of cancer, including breast cancer, and a variety of compounds produced by plants have been investigated for their anti-cancer activity (2-7). Our results have shown that citrus flavonoids inhibit the proliferation both ER- and ER+ human breast cancer cells in vitro (8). In addition to the in vitro studies, we have previously reported that giving orange juice or naringin (the glycoside form of the flavonoid, naringenin, in grapefruit) to rats delayed the development of mammary tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA) (8). In a more recent experiment, in which MDA-MB-435 ER- human breast cancer cells were injected into the mammary fat pads of nude mice, giving them orange juice or grapefruit juice instead of water was found to reduce the incidence of tumors at the site of injection by more than 50% and to inhibit markedly their ability to metastasize to the lymph nodes and lungs (9). The constituent flavonoids from orange or grapefruit juice appeared to be less effective inhibitors of cancer development and metastases in this experiment. Both orange and grapefruit juice contain other bioactive components (Table 1). These include the limonoids, which are one of the two bitter principles found in citrus fruits, including oranges, grapefruits, lemons and limes (10-12). They are also present as glucose derivatives in mature fruit tissues and seeds, and are one of the major secondary metabolites present in citrus. Citrus limonoids were observed to inhibit the proliferation of human breast cancer cells more effectively than the flavonoids (9) and may be largely responsible for the anti-cancer effects of the juices. Our interest in limonoids began with the observation that orange and grapefruit juice inhibited the growth and metastases of human breast cells injected into the mammary fat pad of nude mice and that this inhibition was not completely due to their constituent flavonoids (9). Limonoids have been shown to have anti-cancer activity (13-16). Nomilin reduced the incidence of and number of chemically-induced forestomach tumors in mice when given by gavage (14). Addition of nomilin and limonin to the diet inhibited lung tumor formation in mice and topical application of the limonoids was found to inhibit both the initiation and promotion phases of carcinogenesis in the skin of mice (15).

BODY

Studies With Human Breast Cancer cells in Culture

Cell Culture: MDA-MB-435 estrogen receptor-negative human breast cancer cells were maintained at 37°C in minimum essential medium (alpha modification) containing 3.7 g of sodium bicarbonate per litre, supplemented with 10% v/v fetal calf serum, in a humidified atmosphere of 5% carbon dioxide. Stock cultures were seeded at a density of 2×10^5 cells and allowed to multiply for 48-72 hours.

MCF-7 estrogen receptor-positive human breast cancer cells were maintained in minimum essential medium (alpha modification) containing 3.7 g of sodium bicarbonate supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 10 µg/mL insulin and 1% v/v fungizone (antibiotic/antimycotic, 10 000 units/mL penicillin G sodium, 10 000 µg/mL streptomycin sulphate and 25 µg/mL amphotericin B in 0.85% saline). Cells were grown to confluence at 37°C in a humidified atmosphere containing 5% carbon dioxide and will be passaged weekly using 0.25% trypsin.

Experiments on cell proliferation: The effects of each of the limonoids on the proliferation of MDA-MB-435 ER- and MCF-7 ER+ human breast cancer cells were examined by determining the incorporation of [^{3}H] thymidine into growing cells. MDA-MB-435 cells were plated at a density of 2×10^4 cells/well in 96-well, flat-bottomed tissue culture plates in a total volume of 200 µL of medium and incubated at 37°C, with or without the test compounds. The plates were incubated for 48 hours at 37°C and [^{3}H] thymidine was then added to determine the number of dividing cells at each concentration. The cells were reincubated for 4 hours, after which the medium and excess radiolabel were removed. The cells were trypsinized and harvested onto a glass fiber filter paper, and the radioactivity was counted. The percentage of dividing cells was determined by comparing the number of disintigrations per minute of the treated cells (average of 3 wells/concentration) with that obtained for the control cells. The concentrations at which 50% growth inhibition occured was determined (IC_{50}) for each compound (17). We initially tested the effects of limonin, nomilin, limonin glucoside, and a glucoside mixture (limonin-30%, nomilinic acid-12.7%, nomilin-12.7%, obacunone-8.2%, deacetylnomilin-6.6%, deacetylnomilinic acid-8.7%) from citrus on the proliferation of MDA-MB-435 ER- and MCF-7 ER+ cells. The IC_{50} values for each limonoid are given in Table 2. In ER- cells, the most potent inhibitor of these limonoids is the glucoside mixture having an IC_{50} of 0.08 ug/mL followed by nomilin and limonin. Limonin glucoside was the least effective having an IC_{50} of 75 ug/mL. These limonoids inhibited ER+ cells more effectively having lower IC_{50}s (Table 3). Both nomilin and the glucoside mixture were the most effective having an IC_{50} of 0.05ug/mL (Table 3). We have also tested the effects of a number of naturally occurring and synthetic limonoids for their effect on cell proliferation. In ER- cells, limonin methoxime and deacetylnomilin were the most effective inhibitors having IC_{50}s of 0.02 and 0.07 ug/mL respectively (Table 4, Figure 1). In ER+ cells, deacetylnomilin, obacunone and methyl nomilinate were the most effective inhibitors of proliferation having IC_{50}s of 0.005, 0.009 and 0.01 ug/mL respectively (Table 4, Figure 2). Tamoxifen is a drug widely used in the treatment of hormone

responsive breast cancers and acts mainly by competing with estrogen for its receptor. The IC50s for tamoxifen is 90 μg/mL in ER- cells and 0.04 μg/mL in ER+ cells. Our data indicate that citrus limonoids are potent inhibitors of both cell types and may act via an estrogen receptor-independent pathway. Nomilin and the glucoside mixture are comparable in their inbition of MCF-7 cells to tamoxifen. However, deacetylnomilin, obacunone and methyl nomilinate are more effective in inhibiting ER+ cells than tamoxifen (Table 4).

Viability of Cells: The cytotoxic effects of the limonoids were investigated using the MTT assay (18). In this assay, a tetrazolium salt, 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide (MTT), is reduced to a blue formazan product by mitochondrial dehydrogenases that are active in viable living cells. The intensity of the blue colour that develops is a measure of cell viability.

MDA-MB-435 and MCF-7 cells (8×10^4 cells/well) were seeded in 96-well, flat-bottomed tissue culture plates with various concentrations of limonoids in a total volume of 200 μL/well of medium. Forty-eight hours later, MTT (25 μL of 5 mg/mL) will be added to each well. After three hours, 100 μL of extraction buffer, consisting of 20% SDS, dissolved in a 1:1 dimethylformamide:water solution at pH 4.0, will be added. The blue color formed will be measured at 570 nm in a Dynatech MRX Micoplate Reader. The percentage of cells surviving will be determined by comparing the absorbance of the treated cells with that of the control.

The concentrations of the limonoids that gave 50% cell death (lethal concentration, LC_{50}) was greater than the IC_{50} concentrations in all cases, indicating that the antiproliferative activity of the compounds was not due to nonspecific cytotoxicity (Tables 2 & 3, Figures 1 & 2).

Experiments on Cell Growth: The effect of limonoids on the growth of both types of cells was also studied. MDA-MB-435 and MCF-7 cells were plated at 1×10^4 cells/dish in 60 mm dishes, with or without the test compounds at their IC_{50} concentration (determined in the proliferation assays) in a total volume of 7 mL. The cells were removed by trypsinization at the specified times and counted using a hemocytometer (17).

The ability of limonoids to suppress MDA-MB-435 and MCF-7 cell growth was evident when the cells were grown in the presence of the limonoids at their IC50 values for 10 days, as illustrated for nomilin in figure 3. The effects were clearly apparent after 2 days of treatment, but cell growth was impeded over the entire period of 10 days.

TABLE 1: Average amounts of bioactive compounds in orange juice and grapefruit juice (mg/L).

COMPONENT	ORANGE JUICE	GRAPEFRUIT JUICE
Hesperidin	205	85
Naringin	18	246
Methoxylated flavones	6	<5
Limonene	330	330
Limonoid glucosides	366	198
Limonin glucoside	209	137
Limonin	3	10
Total carotenoids	18	59
Hydroxycinnamic acids	80	89
Ascorbic Acid (Vit. C)	284	250

TABLE 2: Effect of Limonoids on the Proliferation and viability of MDA-MB-435 Estrogen Receptor-Negative Human Breast Cancer Cells in Culture.

LIMONOID	IC_{50} (μg/mL)	LC_{50} (μg/mL)
Limonin glucoside	75	500
limonin	12	80
nomilin	0.4	3
glucoside mixture	0.08	6

TABLE 3: Effect of Limonoids on the Proliferation and viability of MCF-7 Estrogen Receptor-positive Human Breast Cancer Cells in Culture.

LIMONOID	IC_{50} (μg/mL)	LC_{50} (μg/mL)
Limonin glucoside	35	125
limonin	2	63
nomilin	0.05	2
glucoside mixture	0.05	4

TABLE 4: Effect of Limonoids on the Proliferation MDA-MB-435 Estrogen Receptor-Negative Human Breast Cancer Cells in Culture.

LIMONOID	IC_{50} (μg/mL)
limonin methoxime	0.02
deacetylnomilin	0.07
deoxylimonin	0.78
nomilin glucoside	0.78
isoobacunoic acid	1.95
7-a obacunol	3.13
ichangin	3.13
limonol	3.13
Obacunone	3.13
nomilinic acid glucoside	3.13
obacunone glucoside	6.25
limonin carboxymethoxime	10.3
methyl deacetylnomilinate	12.5
methyl deoxylimonate	25.0
methyl nomilinate	25.0
methyl isolimonate	25.0

TABLE 5: Effect of Limonoids on the Proliferation of MCF-7 Estrogen Receptor-positive Human Breast Cancer Cells in Culture.

LIMONOID	IC_{50} (μg/mL)
deacetylnomilin	0.005
Obacunone	0.009
methyl nomilinate	0.01
7-a obacunol	0.15
nomilin glucoside	0.78
isoobacunoic acid	0.78
nomilinic acid glucoside	1.95
obacunone glucoside	1.95
limonin carboxymethoxime	2.50
deoxylimonin	2.50
methyl deacetylnomilinate	2.95
ichangin	3.13
limonin methoxime	3.13
limonol	6.25
methyl deoxylimonate	12.5
methyl isolimonate	12.5

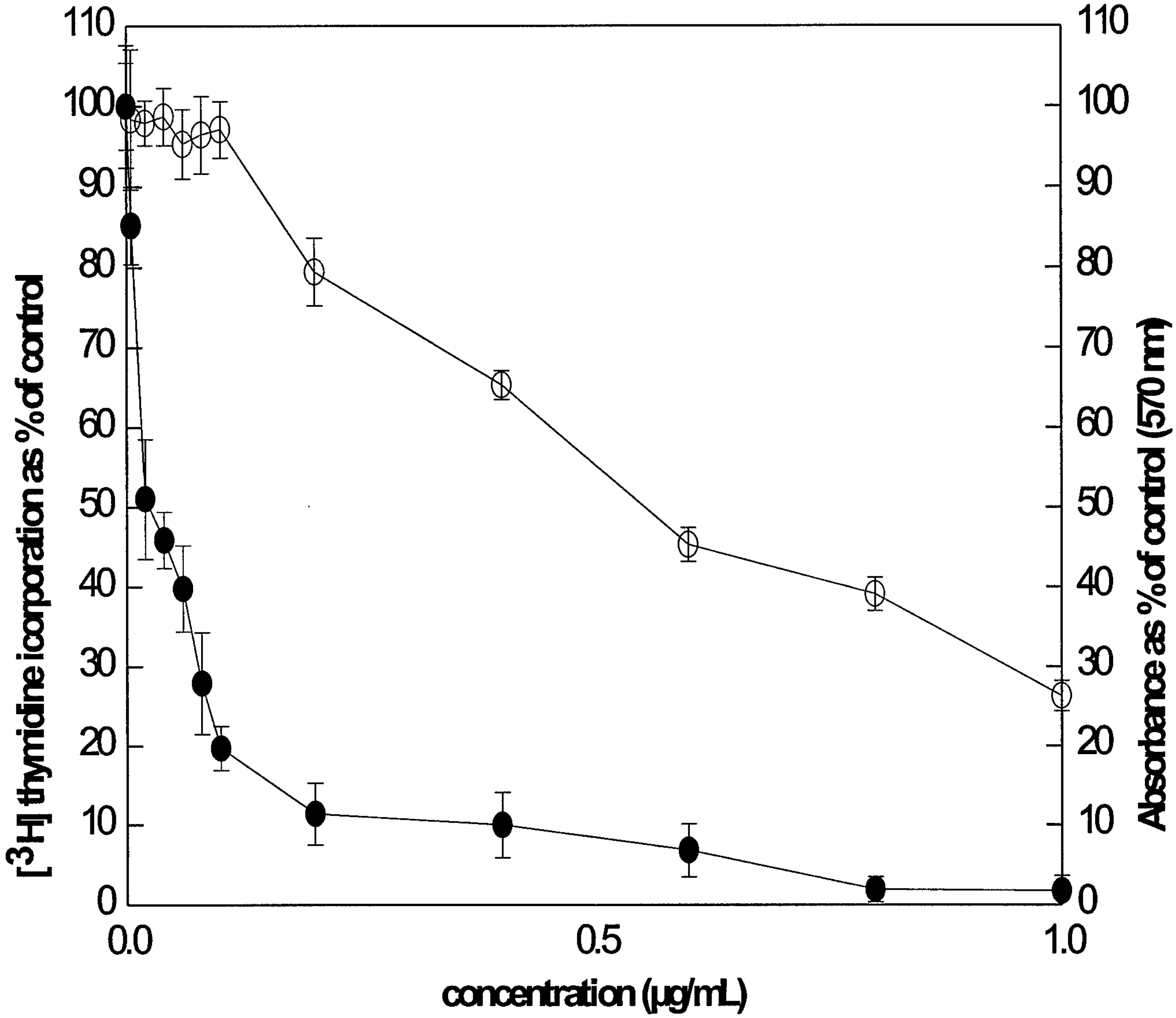

Figure 1: Effect of limonin methoxime on the proliferation (filled circle) and survival (open circle) of MDA-MB-435 estrogen receptor-negative human breast cancer cells in culture as determined by the incorporation of [^{3}H] thymidine and MTT respectively. Results are the averages of 3 separate experiments SEM.

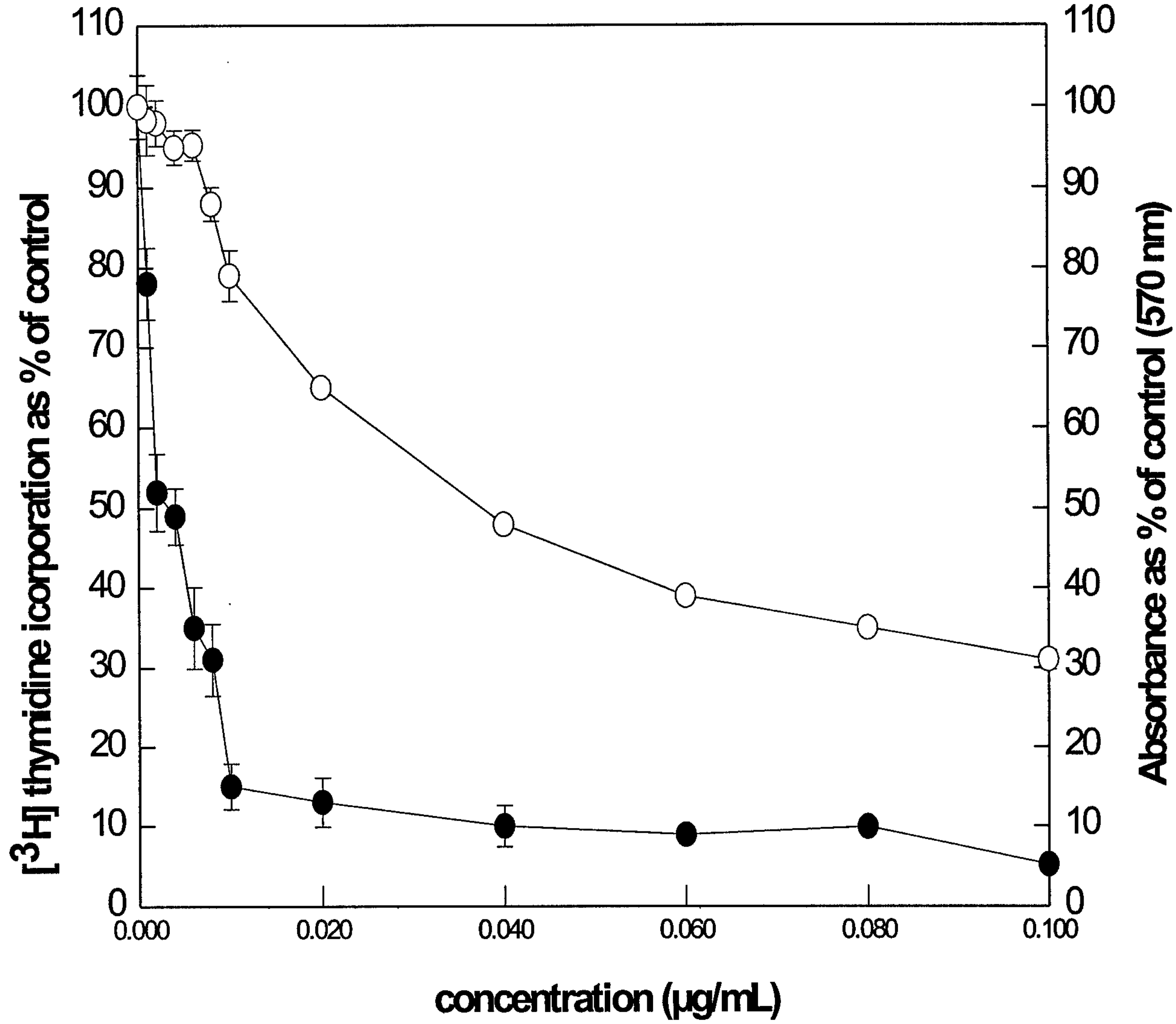

Figure 2: Effect of deacetylnomilin on the proliferation (filled circle) and survival (open circle) of MCF-7 estrogen receptor-positive human breast cancer cells in culture as determined by the incorporation of [^{3}H] thymidine and MTT respectively. Results are the averages of 3 separate experiments SEM.

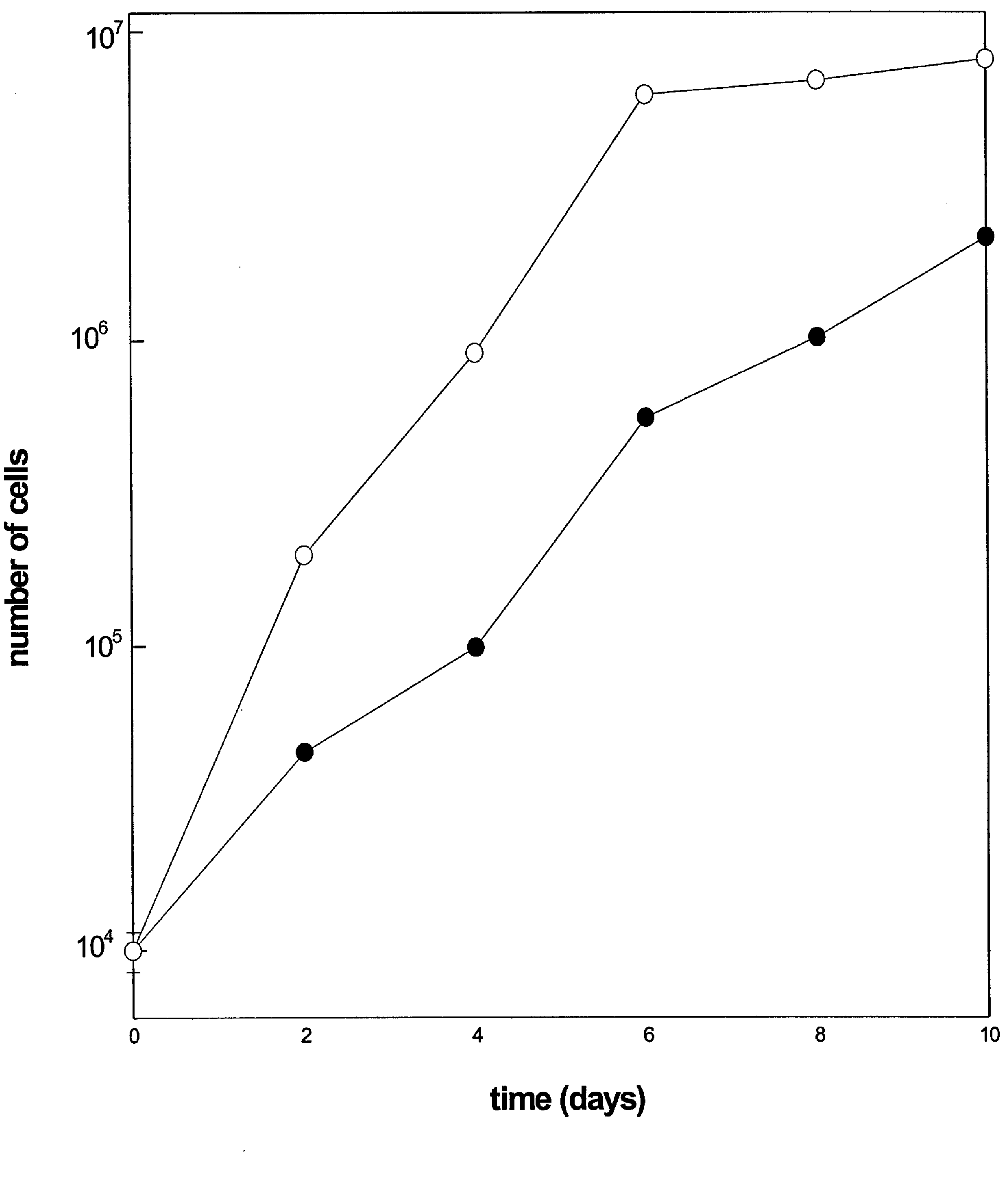

Figure 3: Growth of MDA-MB-435 estrogen receptor-negative human breast cancer cells in presence (filled circle) and absence (open circle) of nomilin at the concentration that inhibited cell proliferation by 50%. Results are the averages of 3 separate experiments SEM.

Animal Studies

Maximum Tolerated dose: Groups of nude mice NCR nu/nu were maintained in a pathogen free animal facility. After a period for acclimitization, they were placed on test diets containing limonin or a glucoside mixture at the initial level of 0.5% by weight. The animals were monitered daily for changes in weight, activity, mobility, and overall health. They were observed for 2 weeks and if no signs of toxicity were observed the level of limonoids in the diet was doubled for other groups of animals. This doubling was repeated until signs of toxicity appeared. The dose ranged from 0.5% to 8% for the glucoside mixture and limonin and 0.5% to 2% for nomilin by weight. At autopsy, liver samples were examined histologically for signs of toxicity.
No significant weight loss was observed for the glucoside mixture at the 8% level. However, histological examination of the liver samples showed signs of early degeneration at 8% and 4% levels. The groups receiving limonin exihibited weight loss and signs of inactivity at the 8% level. Also, liver toxicity was observed at 4% levels for limonin. Based on these findings, we concluded that the maximum tolerated dose is 2% by weight for limonin and 4% by weight for the glucoside mixture. The groups receiving nomilin had significant weight loss and inactivity as well as signs of liver toxicity at the 2% level. It is also important to note that the duration for these studies was short due to availability of limonoids.

Drs. Shin Hasegawa and Gary Manners isolated 100 g samples of each of the limonoids for the next animal experiment. The diets for this experiment have been prepared and sterilized. This experiment is now in progress.

Key Research Accomplishments

- A number of naturally-occurring and synthetic limonoids were screened for activity against both ER- & ER+ human breast cancer cells.
- The maximum tolerated dose for limonin, nomilin and the glucoside mixture were established.
- One hundred grams of each of the limonoids were isolated.
- Diets containing maximum tolerated dose prepared and sterilized.
- Animal study investigating the effect of limonoids at their maximum tolerated dose on ER- tumors is now in progress.
- Presented data at American Chemical Society's limonoid symposium, Aneheim, CA, April, 1999.
- Presented data at Reasons for Hope, Canadian Breast Cancer Research Initiative conference, Toronto, ON, June, 1999.